REMARKS

FORMAL MATTERS

Claims 44-46 are pending in this application. Claims 44-46 have been rejected by the Examiner. Applicants have amended these claims to more particularly point out and claim the invention, support for the amendment to claim 45 may be found in Example 2 and the support for the amendment to claim 46 may be found in Example 1. New claim 47 corresponds to old claim 44, except that it is now dependent on claim 45. Applicants have also amended the specification to insert a figure legend, as requested by the Examiner.

WRITTEN DESCRIPTION REJECTION

The Examiner has maintained his rejection of claims 44-46 as allegedly lacking written description support in the specification under 35 U.S.C. § 112, first paragraph. In particular, the Examiner is concerned about the scope of the terms "at least a portion of a *pol* gene," "at least a portion of the nucleic acid sequence of a *pol* gene," or "at least one domain in a *pol* gene of HIV-1_{BRU}." The Examiner argues that the specification shows that Applicants were in possession of the full-length *pol* gene, but allegedly were not in possession of a large genus corresponding to Pol polypeptide fragments, nucleic acid sequences encoding them, or probes capable of hybridizing to the fragments under the recited conditions.

Cannot Limit Invention to the Preferred Embodiments

The Examiner has discounted the general statements in the specification regarding fragments as only referring to specific *gag* and *env* fragments. Applicants believe that the Examiner is attempting to impermissibly limit the general disclosure with details of certain preferred embodiments. Again, the Examiner does not dispute that the specification provides support for (1) the full length *pol* sequence and (2) the concept that fragments were considered to be a part of the invention. The specification provides the full length pol sequence in Figure 6 and describes the concept of fragments generally by stating "the present invention relates to cloned nucleotide sequences homologous or identical to at least a portion of the genomic RNA of HIV-2 viruses and to polypeptides encoded by the same" (see page 4).

Therefore, the specification shows that the inventors considered fragments of all of the disclosed sequences to be part of the invention. Merely because other fragments are specifically described as preferred embodiments does not limit the general disclosure regarding fragments. "A preferred embodiment . . . is just that, and the scope of a patentee's claims is not necessarily or automatically limited to the preferred embodiment." *Amhil Enterprises Ltd. v. Wawa, Inc.*, 81 F.3d 1554, 1559 (Fed. Cir. 1996). Additionally, "the mere repetition in the written description of a preferred aspect of a claimed invention does not limit the scope of an invention that is described in the claims in different and broader terms." *Laitram Corp. v. NEC Corp.*, 163 F.3d 1342, 1348 (Fed. Cir. 1998).

Thus, it is impermissible to ignore the general statements regarding fragments simply because of disclosure regarding preferred fragments.

Analysis in Light of Enzo Biochem, Inc. v. Gen-Probe Inc.

The recent Federal Circuit case, *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956 (Fed. Cir. 2002) considered under what specific circumstances functional language could support the written description and similarly considered nucleic acid probes. In that case, the claims were directed to nucleic acid probes that selectively hybridize to the DNA of *Neisseria gonorrhoeae*, when compared to a similar bacteria *Neisseria meningitides*. The patentee, Enzo, had identified three nucleic acid probes meeting the claim limitations and had deposited those probes. Enzo argued that the claims were supported by the written description because of the disclosed correlation of the function of hybridization with the bacterial DNA. *Id.* at 967. As strains of the two bacteria were publicly available and could be used to identify which probes would meet the limitations of the claims, the Federal Circuit stated that whether the claims were supported by the written description was a factual one and could not be decided against the patentee in summary judgment. In doing so, the Federal Circuit relied on the Written Description Guidelines issued by the U.S.P.T.O. The Guidelines state that written description can be met by

> Show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

Id. at 964 (quoting Guidelines, 66 Fed. Reg. at 1106).

The Federal Circuit continued by describing an example provided in the guidelines of claims to an isolated antibody to a known antigen, given the well defined structural properties of antibodies, the functional characteristics of antibody-antigen binding, and the high level of scientific understanding in that particular field. *Id.* It concluded, before remanding the case, that the written description requirement would be met for all of the patent claims "if the functional characteristic of preferential binding to *N. gonorrhoeae* over *N. meningitidis* were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed." *Id.* The Federal Circuit continued by stating "[b]ecause the claimed nucleotide sequences preferentially bind to the genomic DNA of the deposited strains of *N. gonorrhoeae* and have a complementary structural relationship with that DNA, those sequences, under the PTO Guidelines, may also be adequately described." *Id.* at 968.

Thus, the court remanded the case to determine "whether a reasonable fact-finder could conclude that the claimed sequences are described by their ability to hybridize to structures that, while not explicitly sequenced, are accessible to the public. Such hybridization to disclosed organisms may meet the PTO's Guidelines stating that functional claiming is permissible when the claimed material hybridizes to a disclosed substrate." *Id.*

The present claims provide more structural information on the fragments than the claims in *Enzo* did. Applicants also wish to point out that the claimed probes in *Enzo* were not fragments of a specified nucleic acid sequence. Claim 1, in *Enzo*, was not

limited to completely complementary sequences and no source of nucleic acid was identified for the probes. Similarly, in *Enzo*, claim 4 recited nucleic acid probes derived from a particular sequence as well as mutants and variants of those probes. *Id.* at 961-62. While the Federal Circuit noted that this increased the number of probes falling within the claimed invention, it also acknowledged Enzo's statement that "such broad claim scope is necessary to adequately protect Enzo's invention from copyists who could otherwise make a minor change to the sequence and thereby avoid infringement while still exploiting the benefits of Enzo's invention." *Id.* at 966. The court, again, concluded that this was a factual issue, but did acknowledge Enzo's statement that without reasonable breadth probe claims would be of limited utility.

Here, unlike *Enzo*, the claims provide that the *pol* nucleic acid fragments are fragments of the sequence set forth in Figure 6. Thus, applicants' claims have a limitation that was missing in Enzo. Similar to Enzo, applicants' claims provide that the fragments hybridize to the sequence set forth in Figure 6. Thus, even more structural information is provided by the claimed sequences than the sequences of *Enzo*, as the fragments are all derived from a single sequence. This combination of structural and functional features having a disclosed correlation between that function and a sufficiently described structure is adequate to provide written description support for this invention.

Additionally, plasmids pROD4.8 and pROD35 contain an isolated nucleic acid of HIV-2 having at least a portion of the nucleic acid sequence of a *pol* gene (see pages 13, 14, and Figure 5). The restriction maps of pROD4 and pROD35 are presented in

the enclosed paper: Clavel, Molecular Cloning and Polymorphism of the Human Immune Deficiency Virus Type 2, Nature 324:691-695 (1986). Both plasmids contain at least a portion of the *pol* gene. This shows that the inventors had possession of the claimed nucleic acids having at least a portion of the *pol* gene. See Declaration of Alizon, ¶ 6.

Furthermore, digestion and southern blotting of DNA extracted from infected CEM cells continuously producing HIV-2 (see example 2, page 20) proves that the inventors had possession of fragments of the *pol* gene that they could extract from the agarose gel or nylon membrane. This Example states:

> DNA was extracted from infected CEM cells continuously producing HIV-1 or 2. The DNA digested with 20 ug of PstI digested with or undigested, was electrophoresed on a 0.8% agarose gel, and Southern-transferred to nylon membrane.

For example, *pol* fragments were created in this experiment because there is a PstI restriction site at position 2481 in the sequence provided (see page 38 of this application). See Declaration of Alizon, ¶ 7.

Applicants request that the Examiner withdraw this rejection.

CONCLUSION

Applicants respectfully requests that this Amendment under 37 C.F.R. § 1.116 be entered by the Examiner, placing claims 44-46 in condition for allowance. Applicants submit that the proposed amendments of claims 44-46 do not raise new issues or necessitate the undertaking of any additional search of the art by the Examiner, since all of the elements and their relationships claimed were either earlier claimed or inherent in

the claims as examined. Therefore, this Amendment should allow for immediate action by the Examiner.

Furthermore, Applicants respectfully point out that the final action by the Examiner presented some new arguments as to the application of the art against Applicant's invention. It is respectfully submitted that the entering of the Amendment would allow the Applicants to reply to the final rejections and place the application in condition for allowance.

Finally, Applicants submit that the entry of the amendment would place the application in better form for appeal, should the Examiner dispute the patentability of the pending claims.

In view of the foregoing remarks, Applicants submit that this claimed invention, as amended, is neither anticipated nor rendered obvious in view of the prior art references cited against this application. Applicants therefore request the entry of this Amendment, the Examiner's reconsideration and reexamination of the application, and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: October 5, 2004

By: Rebecca M. McNeill
Rebecca M. McNeill
Reg. No. 43,796

RESPONSE UNDER 37 C.F.R. § 1.116
EXPEDITED PROCEDURE REQUESTED
EXAMINING GROUP 1648
PATENT
Customer No. 22,852
Attorney Docket No. 03495.0050-16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Marc ALIZON et al.	)	Group Art Unit: 1648
	)	
Appln. No.: 09/988,213	)	Examiner: Parkin, J.
	)	
Filed: November 19, 2001	)	Confirmation No. 8195
	)	
For: Nucleic Acids Containing the Human Immunodeficiency Virus Type 2 (HIV-2) Pol Gene And A Method for Producing a Polypeptide Encoded By The Nucleic Acids (Amended Title)	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Dr Marc ALIZON, declare that:

1. I prepared and enclosed my Curriculum Vitae

2. I have read and understood Application Serial No. 09/988,213, including pending claims 44-46, copies of which are attached hereto.

3. I am an inventor of Application Serial No. 09/988,213, or the subject matter contained therein.

4. I understand that the Examiner has rejected claims 44-46 of this application as allegedly lacking written description support in the specification.

5. I have been asked to comment on the disclosure of isolated nucleic acids of HIV-2 having at least a portion of the nucleic acid sequence of a *pol* gene, in Application Serial No. 09/988,213.

6. I understand that plasmids pROD4.8 and pROD35, which are disclosed in this application, contain an isolated nucleic acid of HIV-2 having at least a portion of the nucleic acid sequence of a *pol* gene (see pages 13, 14, and Figure 5). The restriction maps of pROD4 and pROD35 are presented in the enclosed paper: Clavel, Molecular Cloning and Polymorphism of the Human Immune Deficiency Virus Type 2, Nature 324:691-695 (1986). Both plasmids contain at least a portion of the *pol* gene. This shows that the inventors had possession of the claimed nucleic acids having at least a portion of the *pol* gene.

7. Furthermore, digestion and southern blotting of DNA extracted from infected CEM cells continuously producing HIV-2 (see example 2, page 20) proves that the inventors had possession of fragments of the *pol* gene that they could extract from the agarose gel or nylon membrane. This Example states:

> DNA was extracted from infected CEM cells continuously producing HIV-1 or 2. The DNA digested with 20 ug of PstI digested with or undigested, was electrophoresed on a 0.8% agarose gel, and Southern-transferred to nylon membrane.

For example, I understand that *pol* fragments were created in this experiment because there is a PstI restriction site at position 2481 in the sequence provided (see page 38 of this application).

8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: October 5th, 2004 By: _____
Dr Marc ALIZON

Enclosure : 1 CV

Resume of Marc ALIZON

Born 10/12/1957, aged 46.
Directeur de recherche INSERM since 1987 (DR1 since 2003)

Département de Biologie Cellulaire, Institut Cochin, 22 rue Méchain, 75014 Paris.
E-mail: alizon@cochin.inserm.fr Tel. : 01 40 51 64 86.

Training :

2001 : DEA Epistémology, History of Sciences and Techniques, Université Paris VII
2000 : Qualified as Full Professor, sections # 64 (Biochemistry, Molecular Biology) & 65 (Cell Biology)
1987 : Ph. D. in Virology, Université Paris VI
1985 : M.D., Université Paris V
1982 : DEA Pharmacology, Université Paris VI
1981 : Interne des Hôpitaux de Paris

Positions :

1992 - : Team leader, INSERM U.332 (ICGM) then Institut Cochin
1989-91 : Staff scientist, Institut Pasteur, Paris.
1987-89 : Post-doctoral fellow, Whitehead Institute, Cambridge, Etats-Unis.
1987 : Directeur de recherche INSERM (DR2)
1986 : Chargé de recherche INSERM, Unité d'Oncologie Virale, Institut Pasteur, Paris.

Major publications (10) :

1. Alizon M, Sonigo P, Barré-sinoussi F, Chermann JC, Tiollais P, Montagnier L, Wain-Hobson S (1984) Molecular cloning of lymphadenopathy-associated virus. **Nature** 312 : 757-760.
2. Wain-Hobson S, Sonigo P, Danos O, Cole S, Alizon M (1985) Nucleotide sequence of the AIDS virus, LAV. **Cell** 40 : 9-17.
3. Alizon M, Wain-Hobson S, Montagnier L, Sonigo P (1986) Genetic variability of the AIDS virus : nucleotide sequence analysis of two isolates from African patients. **Cell** 46 : 63-74.
4. Guyader M, Emerman M, Sonigo P, Clavel F, Montagnier L, Alizon M (1987) Genome organization and transactivation of the human immunodeficiency virus type 2. **Nature** 326 : 662-669.
5. Dragic T, Charneau P, Clavel F, Alizon M (1992) Complementation of murine cells for human immuno-deficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. **J. Virol.** 66 : 4794-4802.
6. Pleskoff O, Tréboute C, Brelot A, Heveker N, Seman M, Alizon M (1997) Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. **Science** 276 : 1874-1878.
7. Labrosse B, Pleskoff O, Sol N, Jones C, Hénin Y, Alizon M (1997) Resistance to a drug blocking human immunodeficiency virus type 1 entry (RPR103611) is conferred by mutations in gp41. **J. Virol.** 71 : 8230-8236.
8. Pleskoff O, Tréboute C, Brelot A, Heveker N, Seman M, Alizon M (1997) Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. **Science** 276 : 1874-1878.
9. Brelot A, Heveker N, Montes M, Alizon M (2000) Identification of residues of CXCR4 critical for human immunodeficiency virus coreceptor and chemokine receptor activities. **J. Biol. Chem.** 275 : 23736-23744.
9. Labrosse B, Tréboute C, Brelot A, Alizon M (2001) Cooperation of the V1/V2 and V3 domains of human immunodeficiency virus type1 gp120 for interaction with the CXCR4 receptor. **J. Virol.** 75 : 5457-5464.
10. Bär S, Alizon M. (2004) Role of the ectodomain of the gp41 transmembrane envelope protein of human immunodeficiency virus type 1 in late steps of the membrane fusion process. **J Virol.** 78:811-820.

Tutoring of DEA and PhD students (since 2000)

Anne Brelot. PhD Paris VI (october 2000). CR2 INSERM since 2003.
Béatrice Labrosse. PhD Paris VII (october 2000).
Maurice Chelli.. PhD Paris VI (october 2002).
Séverine Bär. DEA Virology Paris VII (2001). PhD to be defended in 2005.
Etienne Simon-Lorière.. DEA Virology Paris VII (2004).

variation of this concept, the actual function of the hormone may be to induce a conformational change of the receptor that exposes a nuclear translocation signal. Amino acid sequences responsible for nuclear translocation have been identified in several nuclear proteins, and appear to be distinct from the actual DNA-binding site[15,16]. In the absence of the hormone this hypothetical nuclear translocation signal would be masked and the receptor protein would be excluded from the cell nucleus. The possible role of receptor phosphorylation in this remains an intriguing question.

Common to all of these models is the concept that the DNA binding domain of the receptor is functional in the absence of hormone. In the case of oestrogens there is evidence supporting a weak association of the steroid-free receptor with nuclear structures[17,18]. Whether the hormone ligand is required for the activation of gene expression that follows DNA binding could be analysed in appropriate cell-free experiments.

We thank Hannes M. Westphal for the monoclonal receptor antibodies and Pierre Formstecher for the dexamethasone affinity matrix. This work was supported by grants from the Deutsche Forschungsgemeinschaft and from the Fond der Chemischen Industrie.

Received 27 May, accepted 23 October 1986.

1. Fuxe, K. *et al. Endocrinology* 117, 1803–1812 (1985).
2. Antakly, T. & Eisen, H. J. *Endocrinology* 115, 1984–1989 (1984).
3. Payvar, F. *et al. Cell* 35, 381–392 (1983).
4. Scheidereit, C., Geisse, S., Westphal, H. M. & Beato, M. *Nature* 304, 749–752 (1983).
5. Beato, M. & Feigelson, P. *J. biol. Chem.* 247, 7890–7896 (1972).
6. Hynes, N. H. *et al. Proc. natn. Sci. U.S.A.* 80, 3637–3641 (1983).
7. Herrmann, W. *et al. C.r. Acad. Sci. Paris* 294, (III), 933–938 (1982).
8. Westphal, H. M., Moldenhauer, G. & Beato, M. *EMBO J.* 1, 1467–1471 (1982).
9. Idziorek, T. *et al. Eur. J. Biochem.* 153, 65–74 (1985).
10. Baxter, J. D. *et al. Proc. natn. Acad. Sci. U.S.A.* 69, 1892–1897 (1972).
11. Beato, M., Kalimi, M., Konstam, M. & Feigelson, P. *Biochemistry* 12, 3372–3379 (1973).
12. Bourgeois, S., Pahl, M. & Baulieu, E. E. *EMBO J.* 3, 751–755 (1984).
13. Joab, I. *et al. Nature* 308, 850–853 (1984).
14. Tymoczko, J. L. & Phillips, M. *Endocrinology* 112, 142–149 (1983).
15. Kalderon, D., Roberts, B. L., Richardson, W. D. & Smith, A. E. *Cell* 39, 499–509 (1984).
16. Richardson, W. D., Roberts, B. L. & Smith, A. E. *Cell* 44, 77–85 (1986).
17. King, W. J. & Greene, G. L. *Nature* 307, 745–747 (1984).
18. Welshons, W. V., Lieberman, M. E. & Gorski, J. *Nature* 307, 747–749 (1984).
19. Maxam, A. M. & Gilbert, W. *Meth. Enzymol.* 5, 499–560 (1980)

Molecular cloning and polymorphism of the human immune deficiency virus type 2

François Clavel, Mireille Guyader, Denise Guétard, Mireille Sallé*, Luc Montagnier & Marc Alizon†

Unité d'Oncologie virale, and CNRS UA1157, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris CEDEX 15, France
* Diagnostics Pasteur, 3 Bd. R. Poincaré, 92350 Marnes, France

We recently reported the isolation of a novel retrovirus, the human immune deficiency virus type 2 (HIV-2, previously named LAV-2), from patients with acquired immune deficiency syndrome (AIDS) originating from West Africa[1,2]. This virus is related to HIV-1, the causative agent of the AIDS epidemic now spreading in Central and East Africa, as well as the USA and Europe (see ref. 3 for review) both by its morphology and by its tropism and *in vitro* cytopathic effect on CD4 (T4) positive cell lines and lymphocytes. But preliminary hybridization experiments indicated that there are substantiated differences between the sequences of the two genomes[2]. Furthermore, the proteins of HIV-1 and HIV-2 have different sizes and their serological cross-reactivity is restricted to the major core protein, as the envelope glycoproteins of HIV-2 are not immunoprecipitated by HIV-1-positive sera[1,2]. We now report the molecular cloning of the complete 9.5-kilobase (kb) genome of HIV-2, the observation of restriction site polymorphism between different isolates, and a preliminary analysis of the relationship of HIV-2 with other human and simian retroviruses.

A series of filter hybridizations of the HIV-2 RNA genome with probes derived from the complete cloned HIV-1 genome, as well as from regions expected to be well-conserved between HIV-1 and 2 (principally from the *gag* and *pol* genes) yielded extremely weak signals, even in conditions of very low stringency hybridization and washing[2]. This made it very difficult to assess by Southern blot analysis the amount of HIV-2 viral and proviral DNA in infected cells. We therefore cloned a complementary DNA (cDNA) to the HIV-2 genomic RNA, to produce a specific hybridization probe. We used the strategy previously developed for the cloning of HIV-1 (ref. 4). An oligo(dT)-primed cDNA first strand was made in the detergent-activated endogenous reaction (using HIV-2 reverse transcriptase) with virions purified from supernatants of CEM cells (a lymphoblastoid $CD4^+$ cell line[5]) infected with the isolate ROD[2] and continuously producing high amounts of HIV-2. A collection of 10^4 M13 recombinant phages was obtained and screened *in situ* with a labelled HIV-2 complementary DNA first strand. As previously done for HIV-1, we looked directly for HIV-2 specific clones, expected to represent an abundant group in the library, but detected only clones corresponding to cellular repetitive DNA. We can now explain this result by the low representation of HIV-2 specific clones in the library (less than 0.5%, compared to the 5–10% obtained in the cloning of HIV-1). Fortunately the high amounts of recombinant DNA produced by M13, its single-stranded nature, and the ability to perform extremely low stringency hybridization (at T_m −42 °C) on replica filters of M13 plaques allowed the successful use of an HIV-1 probe spanning 1.5 kb of the 3′ end of the LAV_{BRU} isolate (Fig. 1*a*) to screen the library. About 50 positive plaques were detected, purified and characterized by end sequencing and cross-hybridizing of the inserts (Fig. 1). The different clones were found to be complementary to the 3′ end of a polyadenylated RNA, having the AATAAA signal about 20 nucleotides upstream of the poly(A) tail, as found in the long terminal repeat (LTR) of HIV-1 (ref. 6). The part of the HIV-2 LTR that we sequenced is only very distantly related to the homologous domain in HIV-1 (Fig. 1*b*). Only ~50% of the nucleotides can be aligned, requiring the introduction of ~100 insertions and deletions. For comparison, the homology of the corresponding domains in HIV-1 isolates from USA and Africa is greater than 95%, with no insertions or deletions (M.A., unpublished data).

The largest insert of this group of M13 clones (E2, 2 kb) was used as a probe to demonstrate HIV-2 specificity in a series of filter hybridization experiments. First, this probe could detect the genomic RNA of HIV-2 but not HIV-1 in stringent conditions (Fig. 2*C*,*D*). Second, positive signals were detected in Southern blots of DNA from cells infected with the ROD isolate as well as other isolates of HIV-2 (Fig. 2*A* and Fig. 4*A*). No signal was detected with DNA from uninfected (not shown), or HIV-1 infected cells (Fig. 2*A*) confirming the exogenous nature of HIV-2. In undigested DNA from HIV-2 infected cells we detect principally a species of ~10 kb, probably corresponding to linear unintegrated viral DNA, and a species with an apparent size of 6 kb, probably the circular form of the viral DNA. Conversely, rehybridization of the same filter with an HIV-1 probe under stringent conditions showed hybridization to HIV-1 infected cells only (Fig. 2*B*).

To isolate the rest of the genome of HIV-2, a genomic library in lambda phage L47.1 (ref. 7) was constructed with a partial Sau3AI restriction digest of the DNA from the CEM cell line

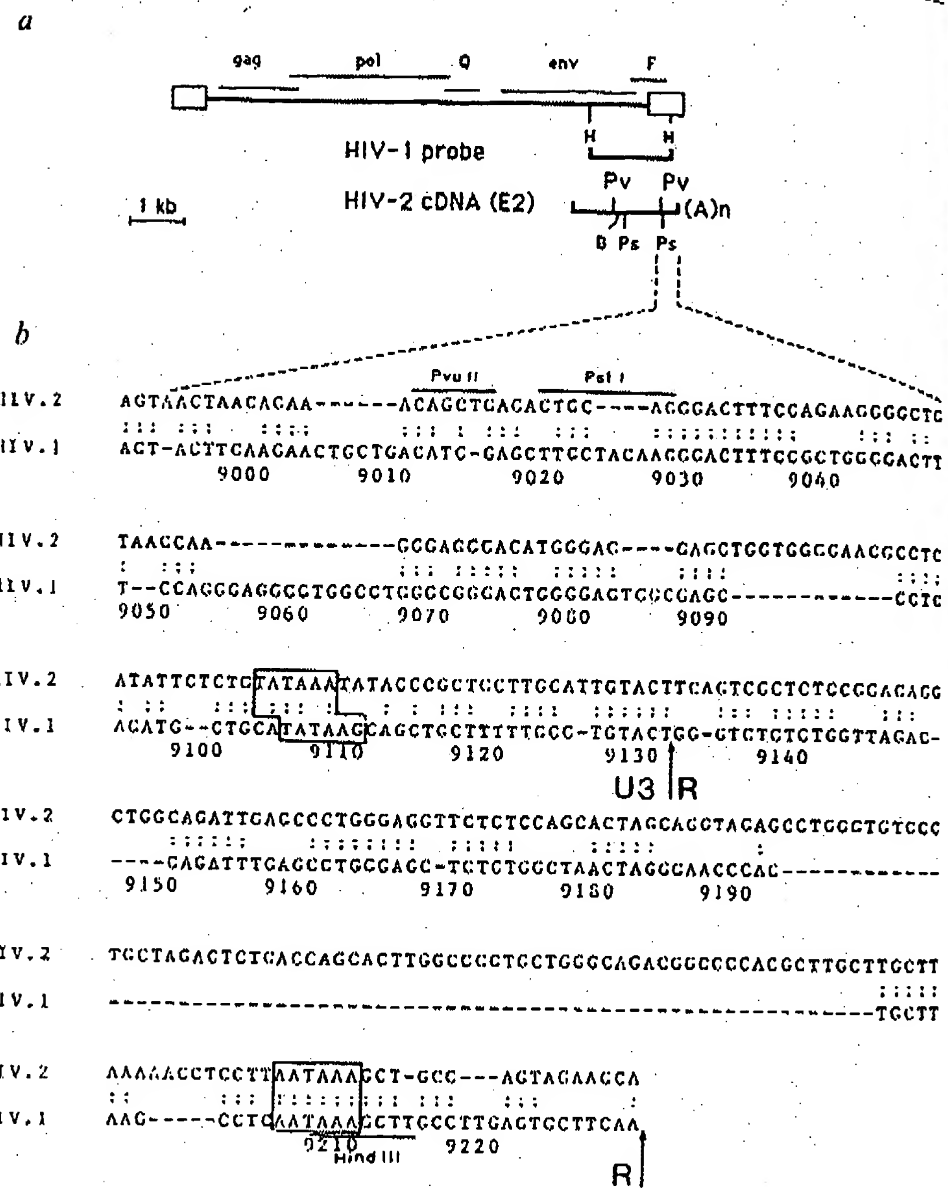

Fig. 1 Cloned complementary DNA (cDNA) from the genomic RNA of HIV-2; *a*, genetic organization of HIV-1, position of the HIV-1 *Hin*dIII fragment used as a probe to screen the cDNA library, and restriction map of the HIV-2 cDNA clone, E2. B, *Bam*HI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II. *b*, partial nucleotide sequence of the LTR of HIV-2. The corresponding region of the HIV-1 LTR[6] was aligned using the Wilbur and Lipman algorithm (window, 10; K-tuple, 7; gap penalty, 3; ref. 14). The U3-R junction in HIV-1 is indicated; poly(A) addition signal and potential TATA promoter regions are boxed.

Methods. HIV-2 virions were purified from 5 litres of supernatant from a culture of the CEM cell line infected with the ROD isolate[2] and a cDNA first strand using oligo(dT) primer was synthesized in a detergent-activated endogenous reaction on pelleted virus resuspended in 1 ml, as described[4]. RNA-cDNA hybrids were purified by phenol-chloroform extraction, and ethanol precipitation. The second-strand cDNA was done by the DNA polymerase I/RNAase H method[15] and blunt-ended with T4 DNA polymerase using a commercial cDNA synthesis kit (Amersham). After attachment of *Eco*RI linkers (Pharmacia), *Eco*RI digestion, and ligation into *Eco*RI-digested dephosphorylated M13 *tg*130 vector (Amersham), a cDNA library was obtained by transformation of the *Escherichia coli* TG1 strain. Recombinant plaques (10^4) were screened *in situ* on replica filters with the 1.5-kb *Hin*dIII fragment from clone J19, corresponding to the 3′ part of the genome of the LAV_{BRU} isolate of HIV-1 (ref. 4), ^{32}P-labelled to a specific activity of 10^9 c.p.m. μg^{-1}. The filters were prehybridized in 5 × SSC, 5 × Denhardt solution, 25% formamide, denatured salmon sperm DNA (100 μg ml^{-1}) at 37 °C, for 4 h and hybridized for 16 h in the same buffer (T_m − 42 °C) plus 4×10^7 c.p.m. of the labelled probe (10^6 c.p.m. per ml of hybridization buffer). The washing was done in 5 × SSC, 0.1% SDS at 25 °C for 2 h. 20 × SSC is 3 M NaCl, 0.3 M Na citrate. Positive plaques were purified and single-stranded M13 DNA prepared and end-sequenced[16].

were screened *in situ* with labelled insert from the E2 cDNA clone. Ten recombinant phages were detected and plaque-purified; three of them were characterized by restriction mapping and Southern blot hybridization with the E2 insert and probes from its 3′ end (LTR) or 5′ end (envelope), as well as with HIV-1 subgenomic probes (HIV-1 probes were used under non-stringent conditions). The λ ROD 4 clone is very likely to contain the complete genetic information of HIV-2, as it carries a 9.5-kb insert and is derived from a circular viral DNA. Two other clones, λ ROD 27 and λ ROD 35 are derived from integrated proviruses and carry a LTR and cellular flanking sequences and a portion of the viral coding sequences (Fig. 3*A*).

The relationship of HIV-2 to other human and simian retroviruses will be more precisely determined by the elucidation of the nucleotide sequence of their genomes, but some preliminary conclusions can already be drawn from hybridization experiments expected to contain the complete HIV-2 genome (Fig. 3*B*). Even at very low stringency (T_m − 42 °C), the hybridization of HIV-1 and 2 is restricted to a fraction of their genomes, principally the *gag* gene (dots 1 and 2), the reverse transcriptase domain in *pol* (dot 3), the end of *pol* and the Q (or *sor*) genes (dot 5) and the F gene (or 3′ ORF) and 3′ LTR (dot 11). The HIV-1 fragment used to detect the HIV-2 cDNA clones contains the dot 11 subclone, which indeed hybridizes well to HIV-2 under non-stringent conditions. Only the signal from dot 5 persists after stringent washing. The envelope gene, but also the region of the *tat* gene and a part of *pol* seem very very divergent. These data, and also the LTR seqeeence (Fig. 1*B*), indicate that HIV-2 is not an envelope variant of HIV-1.

We previously observed that HIV-2 is more related to the simian immune deficiency virus (SIV), than it is to HIV-1 (ref. 2). SIV (also designated simian T-cell lymphotropic ...

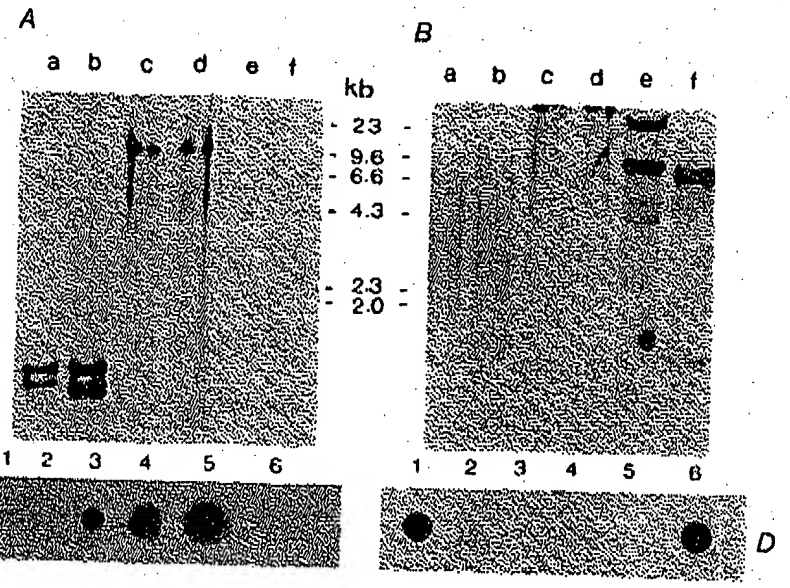

Fig. 2 HIV-2 specificity of the E2 cDNA clone. *A, B*, Southern blots of DNA extracted from CEM cells infected with isolates: HIV-2_{ROD} (a, c) HIV-2_{DUL} (b, d) and HIV-1_{BRU} (e, f); a, b, f, *Pst*I digested; c, d, e, undigested. *C, D*, dot blot hybridization of pelleted virions from CEM cells infected by the HIV-1_{BRU} (1), SIV isolate Mm 142-83 (3), HIV-2_{DUL} (4), HIV-2_{ROD} (5), HIV-1_{RF} (6). Dot 2 is a pellet from an equivalent volume of supernatant from uninfected CEM. *A, C*, Hybridization with the HIV-2 cDNA (E2); *B, D*, hybridization to HIV-1 probe (*Sac*I 9-kb insert from HIV-1_{BRU}, ref. 4).

Methods. DNA was extracted from infected CEM cells continuously producing HIV-1 or 2, and 20 μg of *Pst*I-digested or undigested DNA, was electrophoresed on a 0.8% agarose gel, and transferred to nylon membrane. Virion dot blots were prepared in duplicate as described[2], by pelleting volumes of supernatant corresponding to the same amount of reverse transcriptase activity. Prehybridization was done in 50% formamide, 5×SSC, 5×Denhardt's solution, 100 μg ml^{-1} denatured salmon sperm DNA for 4 h at 42 °C. Hybridization was performed in the same buffer plus 10% Dextran sulphate, and 10^6 c.p.m. per ml of the labelled E2 insert (specific activity 10^9 c.p.m. μg^{-1}) for 16 h at 42 °C. Washing was in 0.1×SSC, 0.1% SDS for 2×30 minutes. After exposition (16 h with intensifying screens), the Southern blot was dehybridized in 0.4 M NaOH, neutralized, and rehybridized in the same conditions to the HIV-1 probe labelled to 10^9 c.p.m. μg^{-1}.

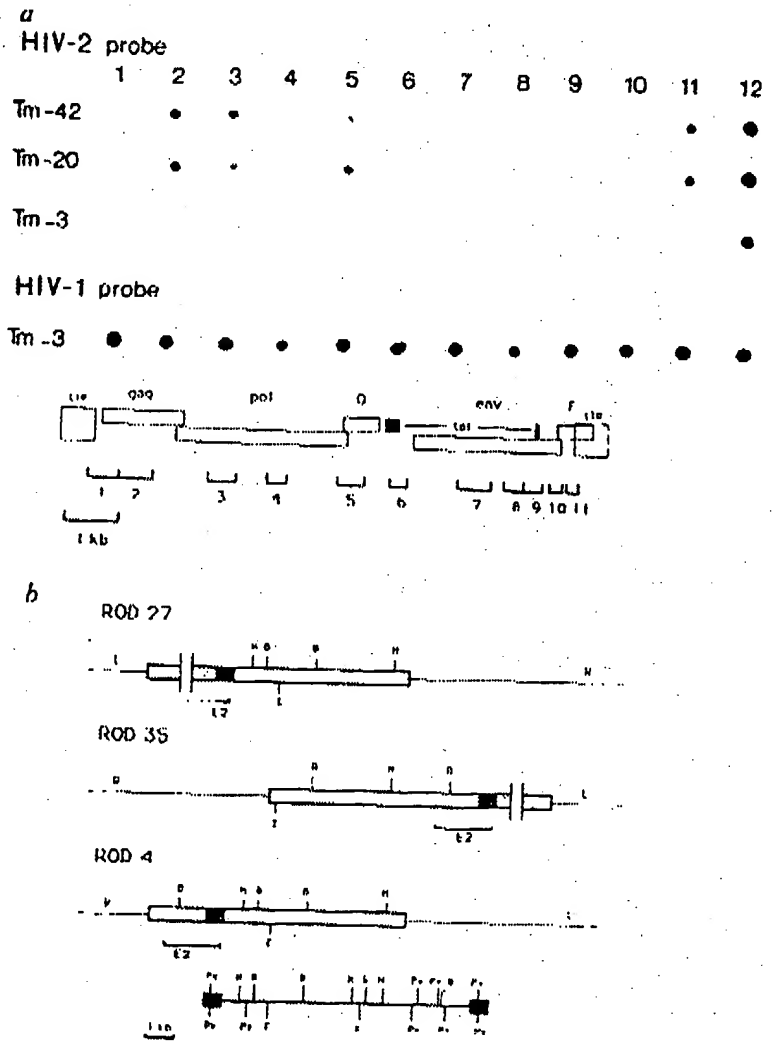

Fig. 3 Restriction map of the HIV-2_{ROD} genomes and similarity to HIV-1. *A*, Organization of three recombinant phage λ clones, ROD 4, ROD 27 and ROD 35; the open boxes represent viral sequence (the LTR are filled), whereas the dotted boxes represent cellular flanking sequences (not mapped); only some characteristic restriction enzymes sites are indicated. ROD 27 and 35 are derived from integrated proviruses whereas ROD 4 is derived from a circular viral DNA. The part of the λ clones that hybridizes to the cDNA E2 is indicated below the maps: a restriction map of the ROD isolate was reconstructed from those of the three λ clones. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sac*I; X, *Xba*I. R, L, right and left *Bam*HI arms of the λL47.1 vector. *B*, 1–11: dots corresponding to the single-stranded DNA form of M13 subclones from the HIV-1_{BRU} cloned genome. Their size and position on the HIV-1 genome, determined by sequencing[6], is shown below the figure. Dot 12 is a control containing λ phage DNA. The dot blot was hybridized in low stringency conditions (as described Fig. 1) with the complete λ ROD 4 clone as a probe, and successively washed in 2×SSC, 0.1% SDS at 25 °C, (T_m − 42 °C), 1×SSC, 0.1% SDS at 60 °C, (T_m − 20 °C), and 0.1×SSC, 0.1% SDS at 60 °C, (T_m − 3 °C) and exposed overnight. A duplicate dot blot was hybridized and washed in stringent conditions (as described in Fig. 2) with the labelled λJ19 clone carrying the complete HIV-1_{BRU} genome[4]. HIV-1 and HIV-2 probes were labelled at the same specific activity (10^8 c.p.m. μg^{-1}).

Methods. DNA from the HIV-2_{ROD}-infected CEM (Fig. 2, lanes a, c) was partially digested with *Sau*3AI; the 9–15-kb fraction was selected on a 5–40% sucrose gradient, and ligated to *Bam*HI arms of the λL47.1 vector[7]. Plaques (2×10^6) obtained after *in vitro* packaging and plating on *Escherichia coli* LA 101 strain were screened *in situ* with the insert from the E2 cDNA clone. About 10 positive clones were plaque purified, and propagated on *E. coli* C600 recBC. The ROD 4, 27 and 35 clones were amplified and their DNA characterized by restriction mapping, and Southern blotting with the HIV-2 cDNA clone under stringent conditions, and *gag-pol* probes from HIV-1 used under non-stringent conditions.

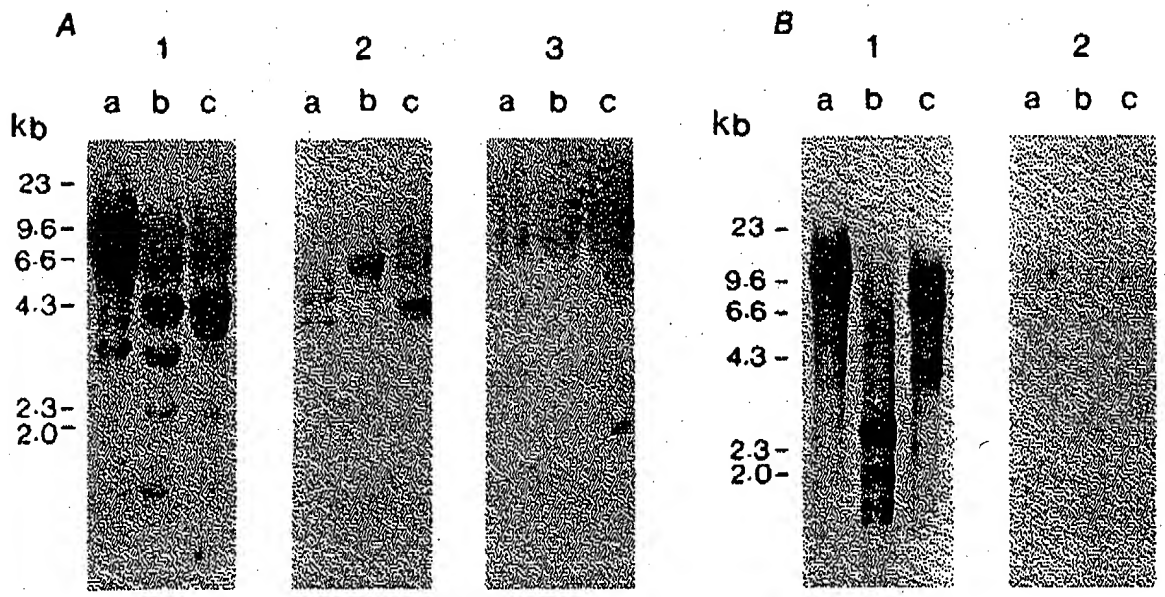

Fig. 4 Restriction map polymorphism in different HIV-2 isolates, and comparison of HIV-2 with SIV. *A*, DNA (20 μg per lane) from CEM cells infected by the isolate HIV-2_{DUL} (panel 1) or peripheral blood lymphocytes (PBL) infected by the isolates HIV-2_{GOM} (panel 2) and HIV-2_{MIR} (panel 3) was digested with: a, *Eco*RI; b, *Pst*I; and c, *Hind*III. Note that much less viral DNA is obtained with HIV-2 isolates propagated on PBL. Hybridization and washing were in stringent conditions, as described in Fig. 2, with 10^6 c.p.m. ml^{-1} of each of the E2 insert (cDNA) and the 5-kb *Hind*III fragment of ROD 4, labelled to 10^9 c.p.m. μg^{-1}. *B*, DNA from HUT 78 (a T lymphoid cell line) cells infected with SIV Mm 142-83; the same amounts of DNA and enzymes were used as for *A*. Hybridization was performed with the same probe as *A*, but in non-stringent conditions, as described (Fig. 1). Washing was for 1 h in 2×SSC, 0.1% SDS at 40 °C (panel 1) and after exposure, the same filter was re-washed in 0.1×SSC, 0.1% SDS at 60 °C (panel 2). The autoradiographs were obtained after overnight exposure with intensifying screens.

infected patients, whereas the serological cross-reactivity of HIV-1 to 2 is restricted to the core proteins[2]. However SIV and HIV-2 can be distinguished by slight differences in the apparent molecular weight of their proteins[2]. In terms of nucleotide sequence, it also appears that the HIV-2 is very close to SIV. The genomic RNA of SIV (provided by Dr R. Desrosiers) can be detected in stringent conditions (Fig. 2*C*) by HIV-2 probes corresponding to the LTR and 3′ end of the genome (E2) or to the *gag* and *pol* genes (data not shown); in the same conditions, HIV-1 derived probes do not detect the SIV genome (Fig. 2*D*). In Southern blots of DNA from SIV-infected cells, a restriction pattern clearly different from HIV-2_{ROD} and other isolates is seen (Fig. 4*B*). All the bands persist after a stringent washing, although the signal is considerably weakened, indicating a sequence homology throughout the genomes of HIV-2 and SIV. It has recently been shown that baboons and macaques could be experimentally infected with HIV-2, providing an interesting animal model for the study of the HIV infection and its preventive therapy (P. Fultz and R. Desrosiers, personnal communication). Attempts to infect non-human primates with HIV-1 have only succeeded in chimpanzees, which are not a convenient model.

We have started a survey of the restriction maps of some of the HIV-2 isolates obtained by our group and from this first study it is already apparent that HIV-2, like HIV-1, undergoes restriction-site polymorphism. Figure 4*A* gives examples of such differences for three isolates, all different one from another and from the cloned HIV-2_{ROD}. It is very likely that these differences at the nucleotide level are accompanied by variations in the amino-acid sequence of the viral proteins, as in the case of HIV-1 (ref. 9).

Recently another group reported the isolation of a human retrovirus from West Africa, apparently highly related to HIV-2 (F.C., unpublished data). HTLV-4 has not been isolated from AIDS patients, and therefore has been postulated to be non-pathogenic[10,11]. We feel that this apparent difference in the pathogenicity of HIV-2 and HTLV-4 is due to their means of discovery. That is, HIV-2 was isolated in a survey of AIDS cases in the West Africa, whereas HTLV-4 was found after a large scale serological screening of populations in Senegal for the presence of anti-HIV-1 antibodies and therefore primarily in healthy individual[11]. As HIV-2 and HTLV-4 infect individuals from neighbouring geographical areas, we think that they will fall into the same group of the HIV family.

Another interest in the characterization of HIV-2 is its possible contribution to the delineation of the domain of the envelope glycoprotein responsible for binding to the surface of the target cells and the subsequent internalization of the virus. This interaction was shown to be mediated by the CD4 molecule itself in the case of HIV-1 (refs 12, 13) and similar studies tend to indicate that HIV-2 uses the same receptor[2]. Thus, although there is wide divergence between the *env* genes of HIV-1 and 2, we may find small homologous domains of the envelopes of the two HIV that could represent candidate receptor-binding site. This site could constitute a target for the attempts of raising a protective immune response against this group of retroviruses.

We thank Dr F. Brun-Vézinet, Hôpital Claude Bernard, Paris, and Dr R. Desrosiers, New-England Regional Primate Research Center, for providing us with the HIV-2_{ROD} and SIV Mm 142-83 isolates, Dr Patricia Fultz, Centers for Disease Control, Atlanta, for information on experimental infections with HIV-2, Drs Pierre Sonigo and Simon Wain-Hobson for their interest in that work, and Dr Michael Emerman for critical reading of the manuscript.

Received 12 November; accepted 24 November, 1986.

4. Alitzon, M. *et al. Nature* 312, 757-760 (1984).
5. Foley, G. E. *et al. Cancer* 18, 522-529 (1965).
6. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. *Cell* 40, 9-17 (1985).
7. Loenen, W. A. M. & Brammar, W. J. *Gene* 10, 249-259 (1980).
8. Daniel, M. D. *et al. Science* 228, 1201-1204 (1985).
9. Alizon, M., Wain-Hobson, S., Montagnier, L. & Sonigo, P. *Cell* 46, 63-74 (1986).
10. Kanki, P. *et al. Science* 232, 238-243 (1986).
11. Barin, F. *et al. Lancet* II, 1387-1389 (1985).
12. Klatzmann, D. *et al. Nature* 312, 767-768 (1984).
13. Dalgleish, A. *et al. Nature* 312, 763-767 (1984).
14. Wilbur, W. J. and Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* 80, 726-730 (1983).
15. Gübler, U. & Hoffman, B. J. *Gene* 25, 263-269 (1983).
16. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* 74, 5463-5467 (1977).

A new way of enhancing the thermostability of proteases

Tadayuki Imanaka, Mitsuyoshi Shibazaki & Masahiro Takagi

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan

Thermostability of enzyme can be enhanced by single amino acid substitutions[1,2]. Recent advances in genetic engineering have made it possible to create novel proteins in a predictable manner where structural information for the protein is available. This 'protein engineering'[3] has already been used to enhance enzyme thermostability[4,5], but it is usually not clear which amino acid substitutions should be made. We consider that the following approach should be helpful in engineering proteins with enhanced thermostability: highly conserved residues should be left unchanged; the sequences of known mesophilic and thermophilic proteins should be used to suggest the kinds of changes likely to increase thermostability[6]; and substitutions should be made that increase internal hydrophobicity and that stabilize helices for strong internal packing. We describe here the use of this approach to alter the thermostability of the thermostable neutral protease of *Bacillus stearothermophilus*, the sequence of which is known[7,8]. Surprisingly we find that a single mutation that decreases thermostability can require two mutations that increase stability to compensate for it. The effects on stability are not additive, suggesting cooperativity.

Comparison of primary structures of enzymes of the same function but different origins is useful to determine the essential regions for activity, because active and/or substrate-binding sites are highly conserved in the homologous regions[9]. Amino acid sequences of four neutral proteases from *B. stearothermophilus*[8], *Bacillus thermoproteolyticus*[10], *Bacillus subtilis*[11] and *Bacillus amyloliquefaciens*[12] were therefore aligned and compared (Fig. 1).

The amino acid sequence of thermostable neutral protease from *B. stearothermophilus* was homologous (85%) with that of thermolysin from *B. thermoproteolyticus*. Similarly, the sequences for thermolabile neutral proteases from *B. subtilis* and *B. amyloliquefaciens* were also homologous (89%). In contrast, the homology between the thermostable and thermolabile enzymes was lower (~45%) (Fig. 1). Nine regions were found to be conserved in all four enzymes. These highly homologous regions are likely to be essential for the enzyme activity. In fact, it has been reported that His 234 and Arg 206 are the active and substrate-binding sites of neutral protease (or of thermolysin), respectively[13]. Zn ion is essential for the enzyme activity of thermolysin, and the three protein ligands for Zn (His 145, His 149 and Glu 169)[13] are conserved in all enzymes (Fig. 1). Glu 146, which promotes the attack of a water molecule on the carbonyl carbon of the substrate[13], is also conserved in these enzymes (Fig. 1).

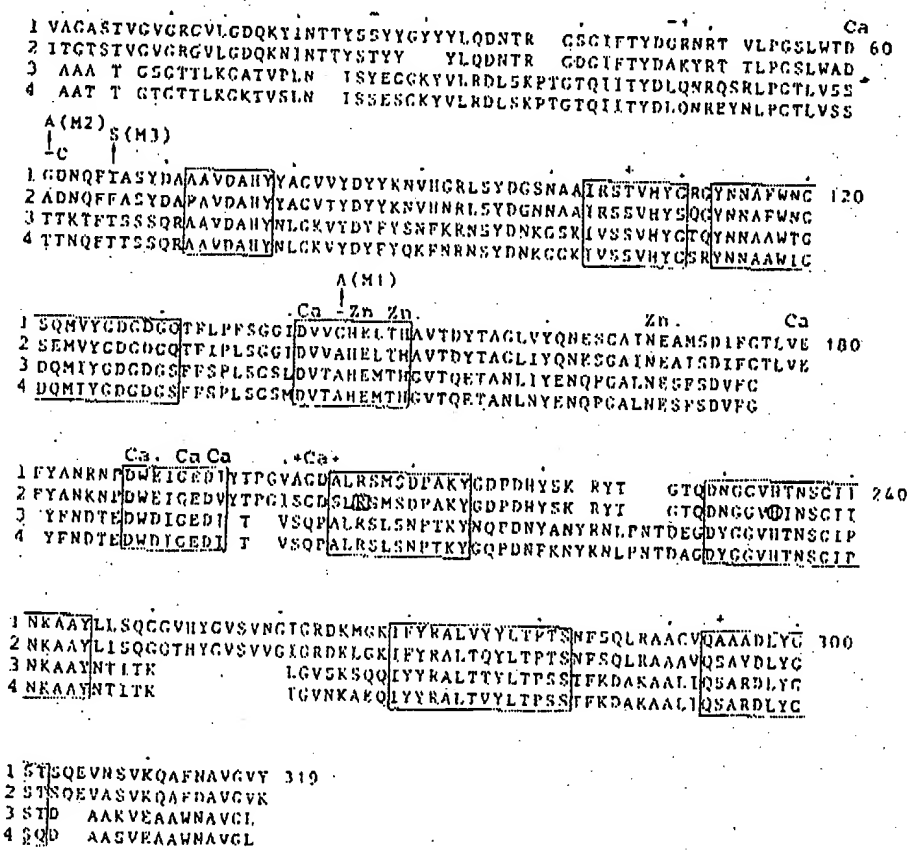

Fig. 1 Comparison of amino acid sequences of various extracellular neutral proteases. Amino acid residues are shown by single letters: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. A blank indicates the absence of corresponding amino acid of this position. Enzyme sources are: 1, *Bacillus stearothermophilus*[8]; 2, *Bacillus thermoproteolyticus*[10]; 3, *Bacillus subtilis*[11]; 4, *Bacillus amyloliquefaciens*[12]. Homologous sequence regions are surrounded by rectangles. Active and substrate-binding sites of thermolysin are indicated by ○ and □, respectively. Protein ligands for Zn and Ca ions for thermolysin are indicated above the sequence. Substitutions expected to enhance or reduce the thermostability of *B. stearothermophilus* protease in comparison with that of thermolysin from *B. thermoproteolyticus* are indicated above the sequence by + or −, respectively. Vertical arrows, amino acid substitutions in *B. stearothermophilus* protease.

1-44. (CANCELED)

45. (CURRENTLY AMENDED) An isolated nucleic acid of HIV-2 having at least a portion of the nucleic acid sequence of a *pol* gene as set forth in Figure 6, wherein the isolated nucleic acid hybridizes with the nucleic acid sequence of a *pol* gene as set forth in Figure 6 in a hybridization solution comprising 50% formamide, 5X SSC, 5X Denhardt solution, 10% dextran sulfate, and 100µg/ml denatured salmon sperm DNA for 16 hours at 42°C with 2 washes for 30 minutes in a solution of 0.1X SSC and 0.1% SDS.

46. (CURRENTLY AMENDED) A nucleic acid of HIV-2 as claimed in claim 45, wherein said nucleotide sequence is a sequence that also hybridizes with at least one domain in a *pol* gene of HIV-1_{BRU} in a hybridization solution comprising 50% formamide, 5X SSC, 5X Denhardt solution, 10% dextran sulfate, and 100µg/ml denatured salmon sperm DNA for 16 hours at 42°C with 2 washes for 30 minutes in a solution of 0.1X SSC and 0.1% SDS.

47. (NEW) A method for producing a polypeptide of HIV-2 having the amino acid sequence, which is encoded by the nucleic acid sequence of claim 45, comprising providing a transformed host containing a DNA coding for the polypeptide and expressing the polypeptide.